Kidney International, Vol. 34 (1988), pp. 209–219

Nephritogenicity of proteoglycans. III. Mechanism of immune deposit formation

HIROFUMI MAKINO, BRIGITTE LELONGT, and YASHPAL S. KANWAR

Department of Pathology, Northwestern University Medical School, Chicago, Illinois, USA

Nephritogenicity of proteoglycans. III. Mechanism of immune deposit formation. Administration of antibody, directed against glomerular basement membrane (GBM) heparan sulfate-proteoglycan, into a presensitized rat results in the induction of membranous nephropathy with subepithelial immune-complex deposits. In this investigation, we examined the mechanisms responsible for the formation of subepithelial immune-complex deposits in the anti-HS-PG model. In initial experiments, the intravenously administered radioiodinated antibody was seen exclusively localized in the regions of the glomerular capillary wall where the subepithelial deposits were observed. To determine their exclusive localization in the subepithelial space, kinetics of movement of the intravenously administered antibody was investigated. The antibody localized in the inner layers of the GBM within a few minutes after its administration. It equilibrated in the inner and outer layers of the GBM in a matter of a few hours. Then, after 24 hours, it gradually disappeared from the inner layers of the GBM and persisted in the outer layers only. The ready clearance of the antibody from the inner layers *may* be related to the differential in the kinetics of lateral intrinsic plasma fluid currents within the GBM. The persistence of heterologous antibody exclusively in the outer layers and the availability of host autologous antibodies probably resulted in the development of immune complex deposits in the subepithelial space. The glomeruli devoid of plasma water currents showed no change in the concentration of the antibody in the inner and outer layers of the GBM or mesangial matrix. Also, no antibody binding was observed with the plasmalemma of either the foot processes or visceral epithelia. The data suggest that the biochemical-biophysical properties of the glomerular capillary wall, in concert with its intraglomerular hemodynamics, most likely played a significant role in the development of subepithelial immune-complex deposits in this model.

Glomerular basement membrane (GBM) is an integral component of renal mammalian vasculature that restricts the transcapillary passage of macromolecules and thus regulates the formation of an ultrafiltrate from blood [1–5]. It is lined inside by the attenuated cytoplasm of fenestrated endothelium and outside by interdigitating epithelial foot processes with intervening slit diaphragms. During the formation of ultrafiltrate, the plasma water, along with proteins, traverses through the endothelial fenestrae, GBM and slit diaphragms, and reaches the urinary space. In doing so, the GBM is constantly exposed to the macromolecules in circulation, including antibodies and immune complexes. These complexes are observed circulating in the blood in many forms of glomerulonephritis [6]. They localize and form immune complex deposits in various regions of the renal glomerulus. In a variety of these nephritides, such as, idiopathic-membranous, post-streptococcal, lupus, and Heymann, the immune complex deposits are seen localized in the outer or subepithelial aspect of the GBM [7–10]. In view of the fact that all the layers of the GBM come in contact with the immune complexes, the mechanisms for the peculiar selective formation of immune deposits in the subepithelial space are poorly understood. It may be that the immune complexes or antibodies, initially localized within the basement membrane, clear with varying rates due to the differential in the kinetics of sweeping plasma water currents in the various layers of the GBM. Given the possibility that the clearance rate may be lower in the outer layer of the GBM, it would favor the retention of antibodies or immune complexes for a prolonged period with the formation of deposits in the subepithelial space ultimately. These considerations led us to investigate the movement of antibodies in various layers of the GBM and the mechanisms involved in the formation of the subepithelial immune-complex deposits. The model utilized for these studies is described in the accompanying paper [11]. In this model, the formation of extensive subepithelial immune-complex deposits was observed as a result of administration of rabbit anti-heparan sulfate-proteoglycan (HS-PG) antibody in animals previously sensitized with rabbit IgG.

Received for publication July 9, 1987
and in revised form December 21, 1987

Methods

In vivo localization of radioiodinated antibody during various stages of immune complex nephritis

Twenty rats were preimmunized with normal rabbit IgG as previously described [11, 12]. Seven days later, they were intravenously administered with radioiodinated anti-HS-PG antibody (50 mg/100 g body wt). They were sacrificed in groups of four at one hour and at 3, 7, 10, and 14 days after the administration of the radioiodinated antibody. The kidneys were flushed with normal saline and fixed by perfusion with Karnovsky's aldehyde fixative. One-mm^3 cortical pieces were made and processed for electron microscopic autoradiography [13, 14]. The control preimmunized animals received radioiodinated normal IgG.

Tracking of the movement of the intravenously administered anti-HS-PG antibody in various layers of the GBM

Preliminary studies were performed to determine the concentration of aldehyde fixative which would cause minimal loss in the reactivity of the intravenously administered antibody. Following the administration of the antibody, the kidneys were perfused with various concentrations (0.001 to 1.0%) of aldehyde fixatives. They were then washed with 0.1 M cacodylate buffer, pH 7.4, and snap-frozen in liquid nitrogen; 4-μm thick cryostat sections were prepared. The sections were stained with anti-rabbit IgG conjugated with fluorescein and examined. Approximately 0.05% glutaraldehyde appeared to be suitable, since at this concentration minimal loss of reactivity was observed without any substantial compromise in the morphology.

Twenty-eight female Fischer rats, each weighing 50 g, were intravenously administered with 25 mg of anti-HS-PG antibody and sacrificed, in batches of four, 5 and 15 minutes, 1 and 6 hours, and 1, 3, and 7 days later. Seven control rats received the same dose of normal rabbit IgG and were sacrificed at the above designated intervals. At the time of sacrifice, their kidneys were fixed by perfusion with 0.05% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for five minutes. After the aldehyde fixative was flushed out of the kidney with 0.1 M cacodylate buffer, 1-mm^3 renal cortical pieces were made and dehydrated in a graded series of ethanol. No osmication or uranyl acetate *en bloc* staining was carried out since these treatments abolished the tissue reactivity of the antibody. During dehydration, the temperature of the tissues was gradually lowered to −20°C. A stepwise infiltration was carried out with Lowicryl K4 medium and finally embedded in this low temperature polymeric resin [15]. A uniform polymerization was carried out under ultraviolet light at −20°C for 24 hours and then at room temperature for 48 hours. Approximately 60-nm thin sections were picked up on 300-mesh nickel grids. The sections were pretreated with 20 mM Tris-buffered saline (TBS), pH 8.2, containing 0.25% bovine serum albumin (BSA). They were then incubated with diluted (1:10) anti-rabbit IgG conjugated with 10 nm or 15 nm colloidal-gold particles (Janssen Pharmaceutica, Belgium) for four hours at room temperature. The dilution was made with TBS buffer containing 0.1% BSA. The grids were then washed with TBS buffer, stained with lead citrate and uranyl acetate, and examined by an electron microscope at an accelerating voltage of 80 KV.

Sixty glomerular capillary loops per time interval were photographed and printed to a final magnification of × 50,000. The number of gold particles present in the inner and outer layers of the GBM were enumerated, tabulated, and expressed as per μm length of the basement membrane. Photographs encompassing mesangium were also taken and printed to a final magnification of × 25,000. For comparative particle-density analysis, gold particles present in the whole width of the GBM and mesangial matrix were enumerated and tabulated. Then the areas of the GBM and mesangial matrix were determined by the point-counting method as previously described [13, 16]. The particle density was obtained by dividing the total number of gold particles by total area points. Then the particle densities on the GBM and mesangial matrices were compared with one another at various time intervals after the administration of the antibody. Finally, a ratio of particle density of the mesangial matrix to the GBM was calculated.

Tracking of movement of antibody in isolated glomeruli

A few experiments were carried out with isolated glomeruli to confirm that the movement of intravenously administered antibody is related to the intrinsic fluid currents in the GBM, that is, when ultrafiltration is proceeding normally. The animals received an intravenous injection of anti-HS-PG antibody (100 mg/100 g body wt). A relatively higher dose of the antibody was used to achieve its sufficient concentration in the GBM at earlier time intervals. Thirty minutes after the administration of the antibody, the kidneys were perfused with oxygenated Hank's-1% BSA solution, severed from the blood vessels, and bisected, and glomeruli were isolated from them [17]. An aliquot of the isolated glomeruli was immersed in an aldehyde fixative (0.03% glutaraldehyde and 0.15% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) for 15 minutes. The remaining glomeruli were suspended in Eagle's minimal essential medium, supplemented with amino acids, 10 mM HEPES, transferrin (5 μg/ml), 1% BSA, penicillin (100 U/ml), and streptomycin (100 μg/ml). The pH of the medium was adjusted to 7.4 by addition of $NaHCO_3$ (0.22 g/100 ml). The glomeruli were cultured for six hours in the same medium with constant shaking and oxygenation. The cultures were terminated by addition of the aldehyde fixative. The aliquots of glomeruli from the time of sacrifice (0 hours) and six hours later were made into pellets in a Beckman microfuge and processed for immunocytochemistry utilizing immuno-gold particles as described above. The six-hour time interval was selected as the terminal point since the glomerular morphology seems to deteriorate beyond this period under culture conditions. Twenty-five micrographs, from each time interval (0 and 6 hours), were photographed and printed to a final magnification of × 25,000. The immuno-gold particles were enumerated on the mesangial matrix and GBM of the same micrograph. The particle density analyses and their ratios (mesangial matrix/GBM) were calculated as described above. Finally, mean and standard deviation for each of the parameters were determined.

Antibody binding studies with isolated glomeruli and GBM

In order to ascertain the reactivity of anti-HS-PG antibody with visceral epithelial cells, the binding experiments were carried out with isolated glomeruli and GBMs, separately and in combination. Three sets of experiments were performed.

First, the antibody binding with isolated glomeruli was investigated. The glomeruli were isolated from kidneys perfused with oxygenated Hank's balanced salt solution [17]. The glomeruli isolated from four kidneys were suspended in Hank's-1% BSA solution with ^{125}I-anti-HS-PG antibody (1 mCi/50 mg/ml) for 10 minutes at 4°C. After incubation, the glomeruli were washed with Hank's-BSA solution, pelleted, and processed for electron microscopic autoradiography [13, 14, 16].

In the second set of experiments, the antibody binding with the GBMs was investigated. The glomeruli were isolated, and basement membrane fractions were prepared by the detergent method [18, 19]. The GBMs were then incubated with radioiodinated antibody and processed.

In the third set of experiments, the isolated glomeruli and GBM were mixed and a suspension prepared. After the addition

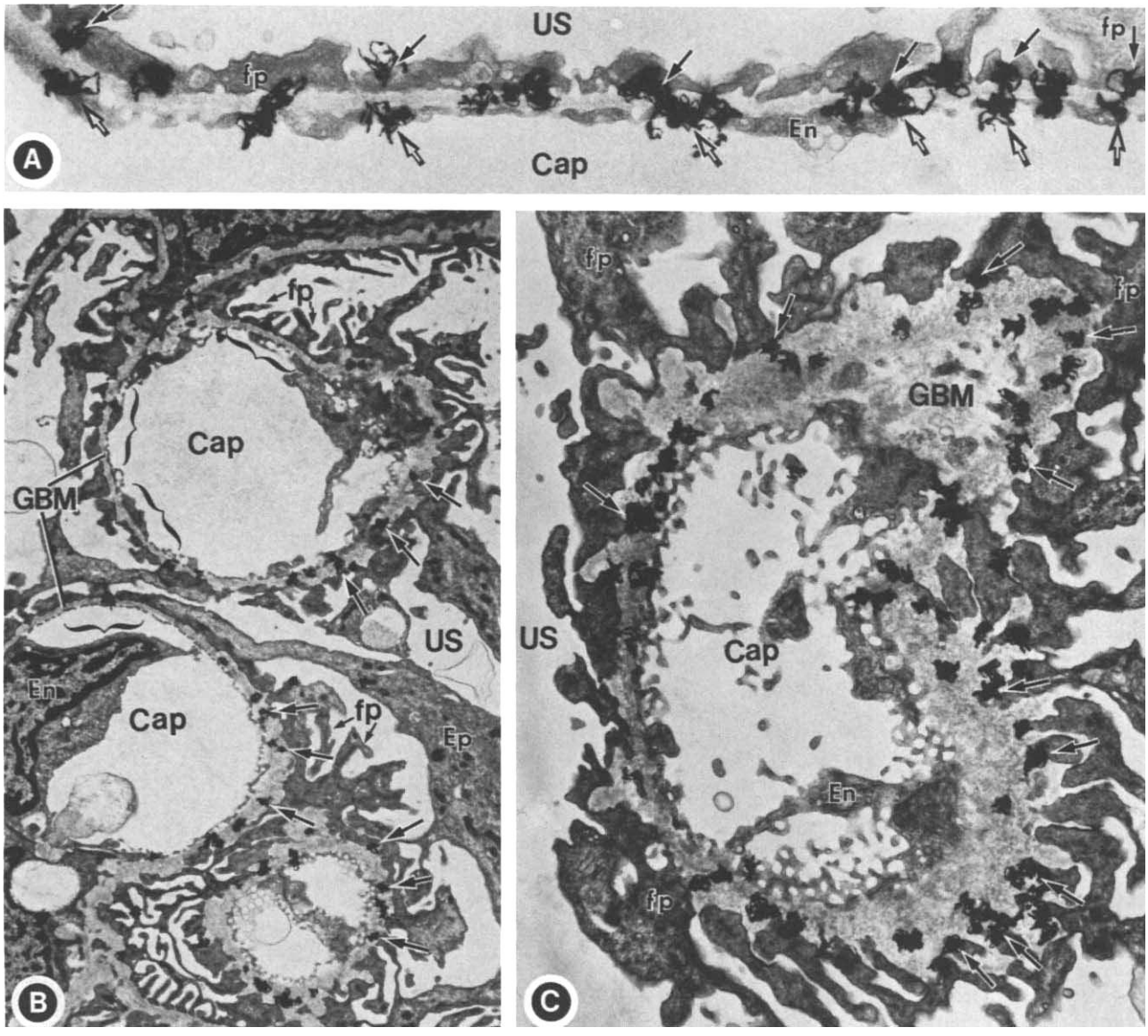

Fig. 1. *Electron microscopic autoradiograms of glomerular capillary loops (Cap) from presensitized rats that received radioiodinated anti-HS-PG antibody and were sacrificed 1 hour (A), 7 days (B), and 2 weeks (C) later.* A. Initially, the antibody (represented as silver grains) is seen uniformly distributed on either side of the basement membrane (arrows). B. One week later, the grains (arrows) are seen only in the regions where the subepithelial immune deposits are observed. The regions devoid of deposits (within brackets) are uniformly thin and are free of radioiodinated antibody. In grazing sections (panel C), the concentration of the antibody (arrows) seems to be proportionately higher in the regions where there are more subepithelial deposits. US, urinary space; Ep, epithelium; fp, foot processes; En, endothelium. A: × 25,000; B: × 6,000; C: × 15,000.

of radioiodinated antibody, the glomerular-GBM suspension was incubated for 10 minutes at 4°C and then washed three times with Hank's-BSA solution. Finally, a pellet was prepared by centrifugation and processed for electron microscopic autoradiography. The total time required for the preparation of glomeruli, incubation with the antibody, and immersion in the glutaraldehyde fixative was estimated to be about 30 minutes. In control experiments, ^{125}I-normal rabbit IgG was used in place of radioiodinated antibody.

Results

Initial observations, which led to the suggestion that HS-PG may be the site where immune complex deposits form in the GBM, are detailed in the data presented in the following section.

In vivo localization of radioiodinated antibody during various stages of immune complex nephritis

The radioiodinated antibody was seen localized to the GBM within a few minutes after its administration. The antibody-corresponding autoradiographic silver grains were seen over the whole width of the GBM at regular intervals (Fig. 1A). Their grain-point centers appeared to reside in the inner and outer layers of the GBMs. This regular pattern of autoradiographic grains persisted for three to four days; thereafter, their distri-

bution became somewhat erratic. A week later, when immune deposits were visualized and irregular thickening of basement membrane ensued, the grains were seen mainly concentrated in the altered regions of the GBMs (Fig. 1 B and C). Their concentration seemed to be proportionately higher in the areas of the GBM having more of the immune deposits (Fig. 1B). Moreover, the point-center of the grains appeared to correspond with the center of the electron-dense deposits, thus suggesting a topological superimposition of the radioiodinated anti-HS-PG antibody over the immune complex deposits. The regions of the GBM devoid of deposits were sparsely studded with the autoradiographic grains. Further affirmation that the HS-PG site may serve as the core-nidus for the formation of an immune complex deposit was obtained in the grazing sections of the capillary loops. In these sections, the autoradiographic grains populated mainly in the outer layers of the GBM where the subepithelial deposits were heavily concentrated (Fig. 1C). The inner layers of the GBM, which were devoid of immune deposits, did not contain any autoradiographic grains. This meant that the immune deposits evolved only in the regions where there is a persistent residence of the intravenously administered antibody, that is, in the outer layers of the GBM. These data indicated that there may be a differential in clearance/persistence/residence of the intravenously administered antibody in various layers of the GBM. The anticipated differences in the clearance rates of the antibody, localized in the inner and outer layers of the GBM, are elucidated in the next set of experiments.

Tracking of the movement of the intravenously administered anti-HS-PG antibody in various layers of the GBM by immunoelectron microscopy

The tissues processed for immuno-electron microscopy were prefixed by a brief exposure to 0.05% glutaraldehyde. At this concentration of aldehyde, no appreciable loss of the antibody reactivity was noted. A reassurable linear pattern of immunofluorescence of the GBM was observed upon staining with anti-HS-PG antibody.

After the administration of IgG fraction of rabbit anti-HS-PG, the antibody was seen exclusively localized in the GBM. The antibody was visualized in the GBMs with the use of immunogold particles. No reactivity towards the immuno-gold particles was observed either on non-glomerular basement membranes or cell surfaces, indicating an absence of antibody in these structures. Similarly, no reactivity was observed in tissues from animals injected with normal rabbit IgG. At early time intervals (5 and 15 min), the particles were seen mainly in the inner layer of the GBM (Fig. 2 A and B). No immunoreactivity was seen in the mesangium (Fig. 3A). With passage of time (1 and 6 hrs), an increase in the number of particles in the GBM was noted. They were seen with almost the same frequency on either sides of the basement membrane (Fig. 2 C and D). A few particles were seen in the mesangium at these intervals. In later time points (1, 3, and 7 days), a gradual decrease in the number of particles was observed (Fig. 2 E and F). The observed decrease was much steeper for the inner layers of the GBM. On day 7, the inner layer was virtually free of reactivity towards the immuno-gold particles (Fig. 2F), and they were exclusively seen in the outer layer or subepithelial region of the GBM. The mesangium had a

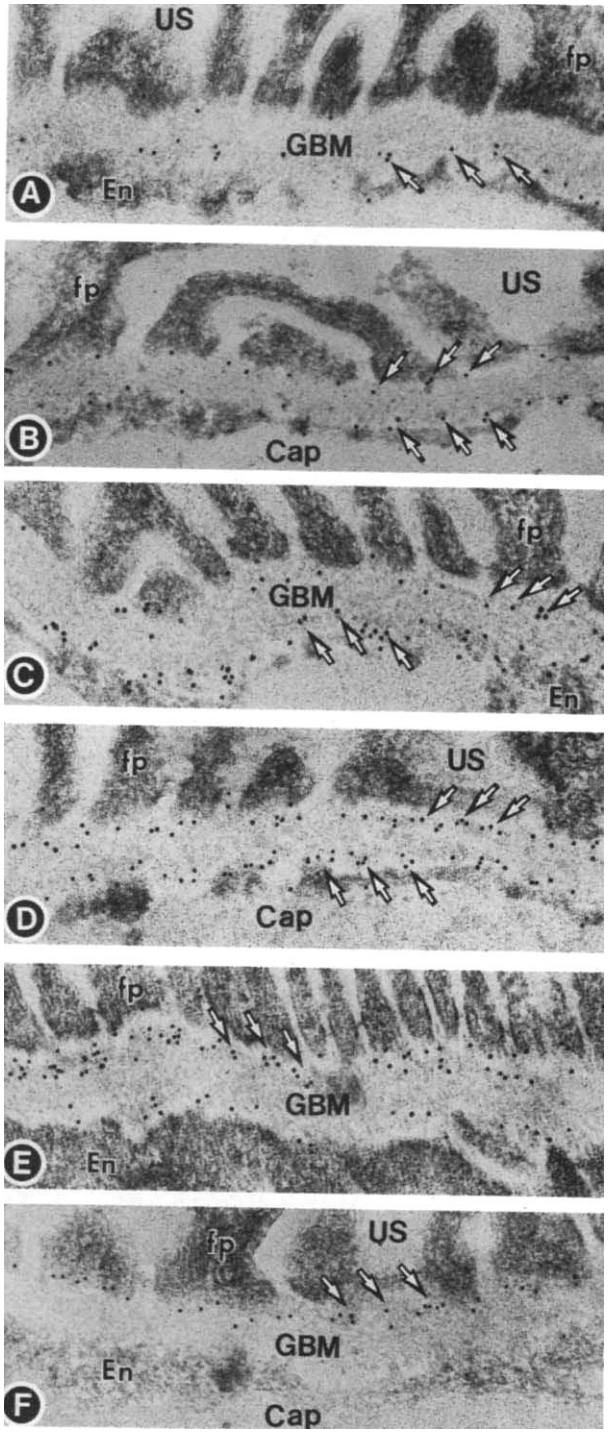

Fig. 2. *Electron micrographs of glomerular capillary loops (Cap) obtained from rats sacrificed 5 minutes* (**A**) *and 15 minutes* (**B**), *1 hour* (**C**) *and 6 hours* (**D**), *1 day* (**E**) *and 7 days* (**F**) *after the administration of the antibody*. The thin sections were prepared on nickel grids and stained with anti-rabbit IgG conjugated to colloidal gold. The antibody is visualized as electron-dense gold particles (arrows). At earlier time points (panels A and B), the antibody (arrows) is seen mainly in the inner layers of the glomerular basement membrane (GBM). After 1 and 6 hours (panels C and D), the antibody is seen equally distributed on both sides of the GBM. At later time points, that is, 1 and 7 days (panels E and F), the antibody is mainly seen in the outer layers of the GBM. US, urinary space; Ep, epithelium; fp, foot processes; En, endothelium. A-F: × 75,000.

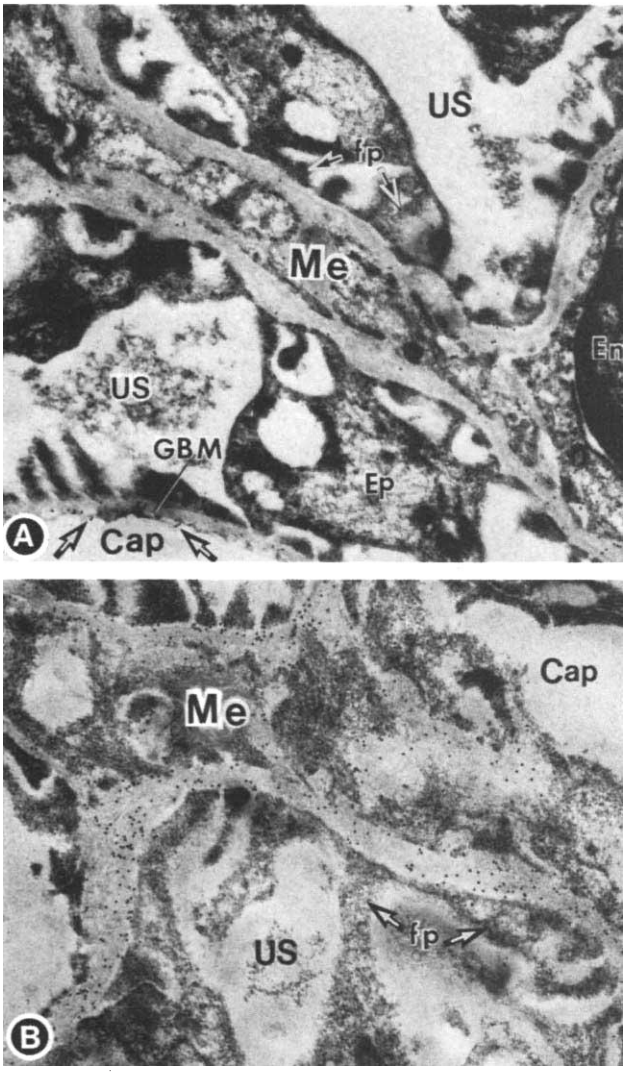

Fig. 3. *Electron micrographs of glomerular mesangium (Me) obtained from rats sacrificed 15 minutes* (**A**) *and 3 days* (**B**) *after the administration of anti-HS-PG antibody.* A. At earlier time point, the antibody (represented by electron-dense colloidal-gold particles) is not seen in the mesangium, while it (arrows) is normally present in the GBM of peripheral capillary loops (Cap). B. At later time point, the concentration of antibody dramatically rises in the mesangium. US, urinary space; Ep, epithelium; fp, foot processes; En, endothelium. A and B: × 15,000.

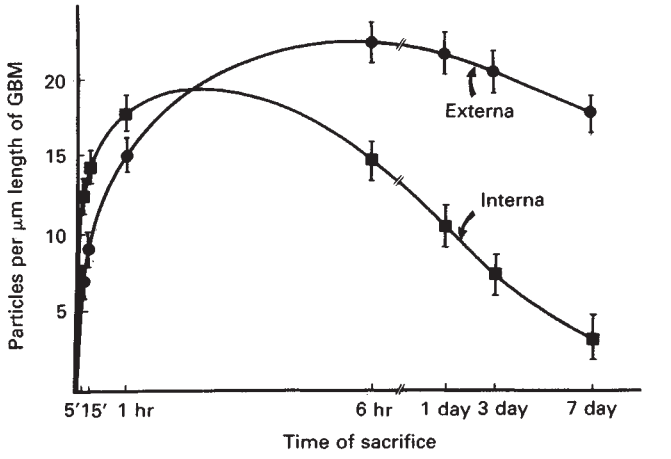

Fig. 4. *Graphic representation of the antibody concentration (represented by immuno-gold particles per μm length of the GBM) in the inner and outer layers of the basement membrane at various time intervals after its administration.*

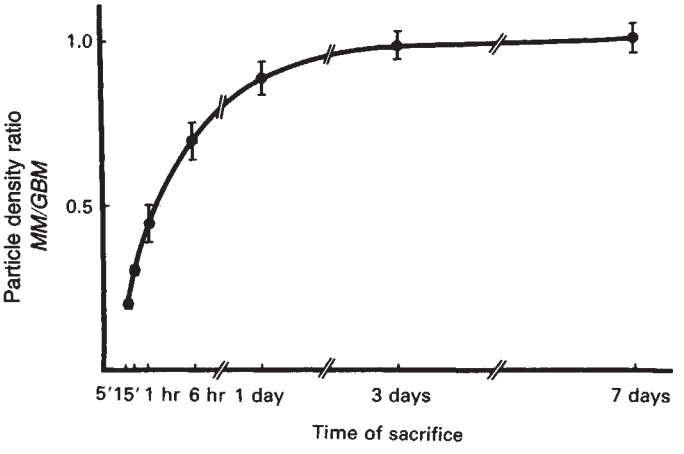

Fig. 5. *Graphic representation of the ratio of the antibody (represented by the immuno-gold particles) in the mesangial matrix (MM) and GBM of the glomerulus at various time intervals after its administration.* Symbols are: (●) externa; (■) interna.

progressive increase in the concentration of the antibody, as evidenced by accentuated reactivity towards immuno-gold particles (Fig. 3B). Figure 4, prepared after enumeration and tabulation of the immuno-gold particles, reinforced the qualitative assessment of the concentration of antibody present at various time intervals in different layers of the GBM. The comparative particle-density analysis revealed a very low mesangial matrix/GBM ratio initially, and it gradually rose to unity by 12 hours (Fig. 5). The ratio remained at unity for the next seven days. This indicated that the mesangial matrix gets saturated with the antibody molecules very early, that is, by 12 hours, and remains saturated for the rest of the investigatory period. An occasional immuno-gold particle was observed over the lysosomes of the mesangial cell.

Tracking of movement of the antibody in isolated glomeruli

In these experiments, the movement of the intravenously administered antibody was investigated in the absence of intraglomerular hemodynamics. Thirty minutes after the administration of the antibody (designated as 0-hour time interval), most of the immuno-gold particles were seen in the GBM, with very few in the mesangial matrix (Fig. 6A). The respective particle densities in the mesangial matrix and GBM were 0.46 ± 0.05 and 3.41 ± 0.04; their ratio (mesangial matrix/GBM) was 0.140 ± 0.024. After six hours of culture, the number of particles in the GBM and mesangial matrix appeared to be the same as seen at the 0-hour time interval. No increase in the mesangial matrix or decrease in the GBM was observed (Fig. 6B). Quantitatively, the respective particle densities for the mesangial matrix and

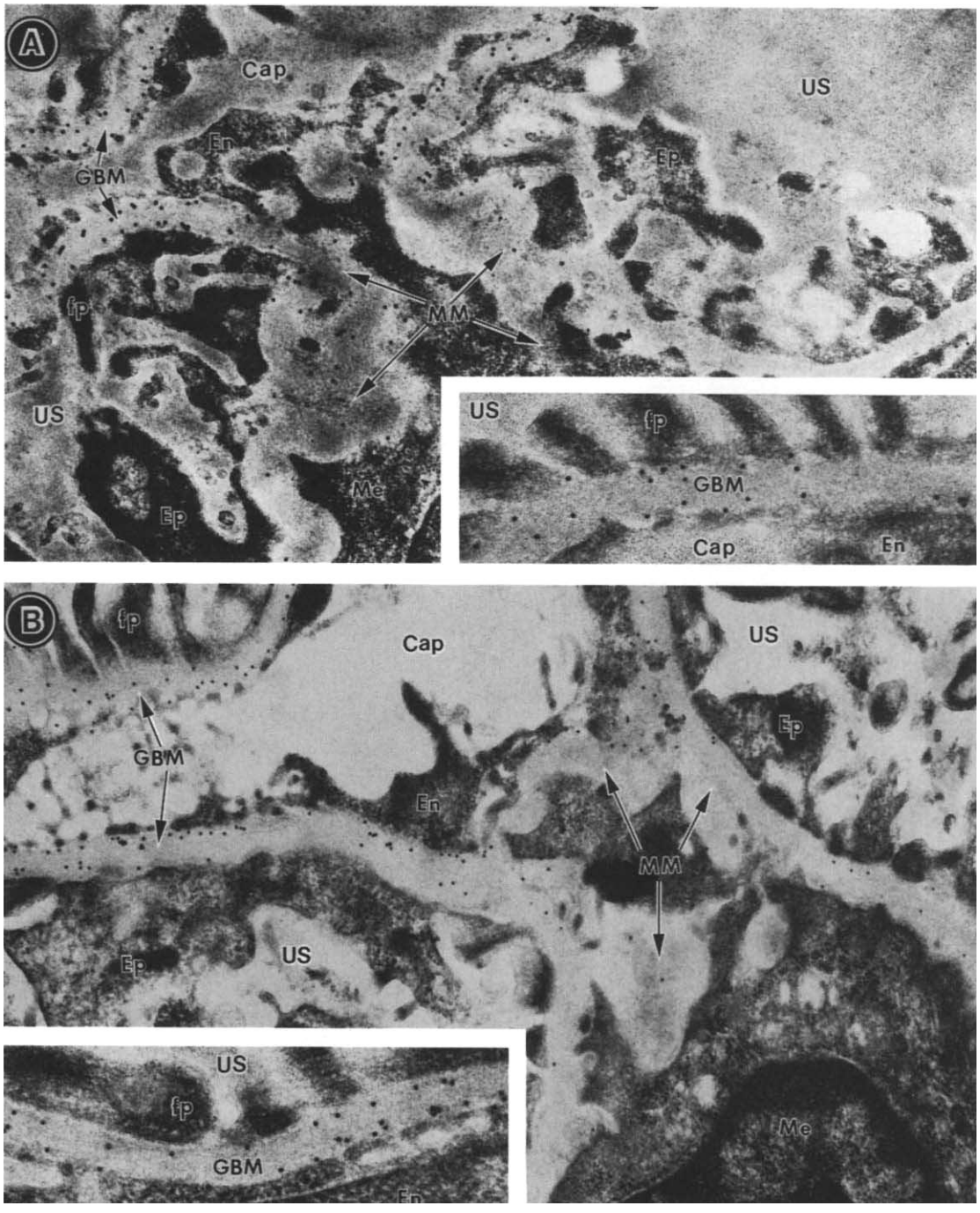

Fig. 6. *Electron micrographs of isolated glomeruli obtained from rat sacrificed 30 minutes after the administration of anti-HS-PG antibody* (**A**)*, and further in vitro culture for 6 hours* (**B**). The thin sections were prepared and reacted with anti-rabbit IgG conjugated to colloidal gold. No significant change in the concentration of the antibody (represented by electron-dense gold particles) is seen, either in the GBM or mesangial matrix (MM), during the 6 hours of the incubation period. US, urinary space; Ep, epithelium; fp, foot processes; En, endothelium; Me, mesangium; Cap, capillary loop. A: × 40,000; B: × 30,000.

GBM were 0.49 ± 0.06 and 3.33 ± 0.39; their ratio was 0.146 ± 0.06. This suggested that no significant movement of the ligand occurs in the extracellular matrices when the kidney is devoid of its intraglomerular hemodynamics, that is, the redistribution of the ligand ceases completely in the absence of ultrafiltration.

From the above experiments, it appears that the mechanisms involved in the formation of subepithelial immune-complex deposits may be regional to the GBM due to its peculiar macromolecular composition. However, in recent years, Dr. Andres and his associates have proposed another hypothesis in

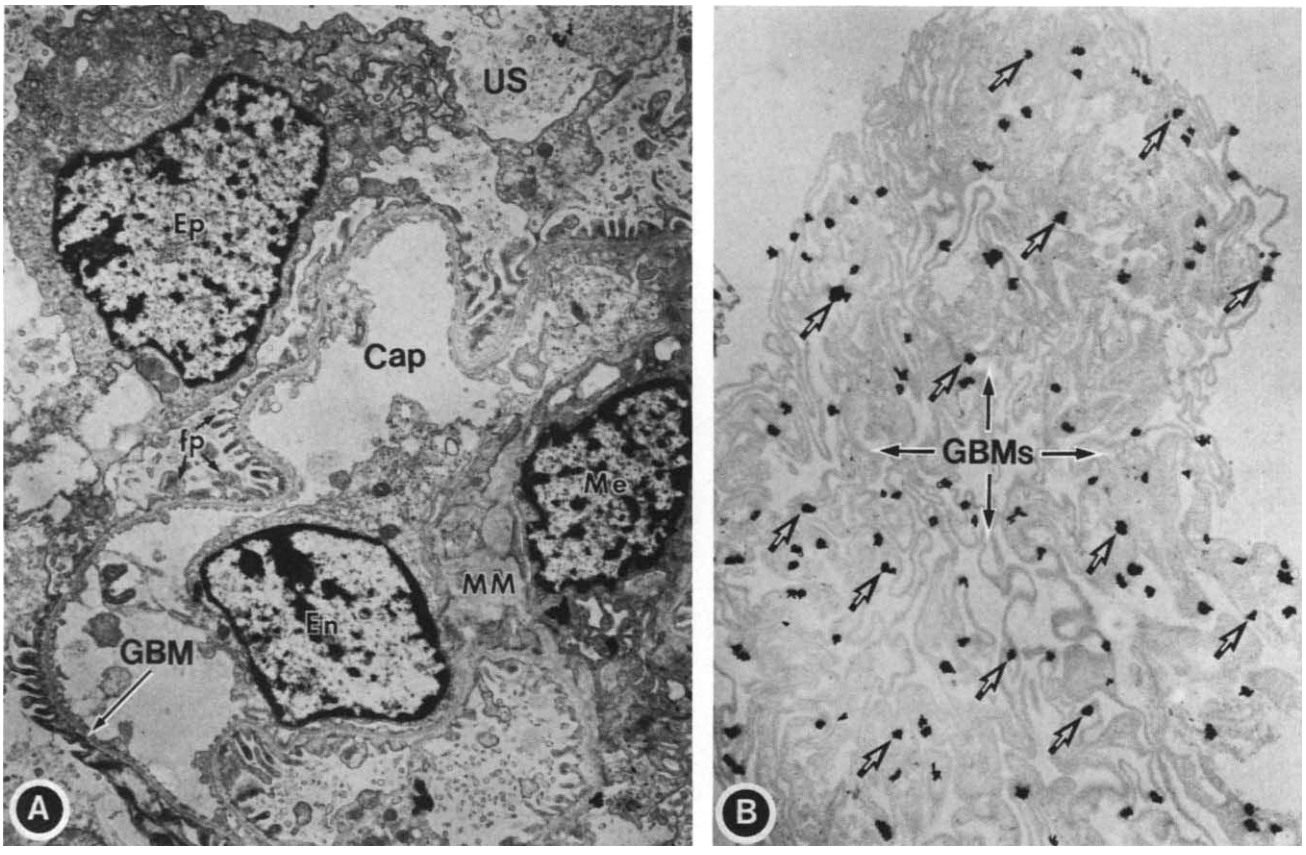

Fig. 7. *Electron microscopic autoradiograms of isolated glomeruli* (**A**) *and GBMs* (**B**) *incubated with radioiodinated anti-HS-PG antibody.* A. The antibody is not seen binding to the visceral epithelium (Ep), and only occasional background grains are observed. B. The antibody (represented by silver grains and indicated by arrows) is seen bound to the isolated GBM loops. US, urinary space; fp, foot processes; En, endothelium; Me, mesangium; MM, mesangial matrix; Cap, capillary loop. A: × 6,000; B: × 5,000.

the formation of subepithelial deposits utilizing the model of Heymann nephritis [20, 21]. According to their hypothesis, the antibodies bind to the podocytes, cause redistribution and aggregation of antigen-antibody complexes at the visceral epithelial cell surface, and then shed into the subepithelial regions where the immune complex deposits are formed ultimately. Here, the key issue pertains to the fact that the antibody must bind to the visceral epithelial cells of the glomerulus in the initial events of Heymann nephritis. To address this issue in our model, the next series of antibody binding experiments were performed, and their results are detailed below.

Antibody binding studies with isolated glomeruli and GBMs

By autoradiography, antibody was not seen bound to the visceral epithelial cells of the isolated glomeruli. Only the decapsulated glomeruli were examined. A very few background grains were visualized over the entire regions of the glomerulus (Fig. 7A). In contrast, a large number of autoradiographic grains was observed over the isolated GBMs (Fig. 7B), indicating the binding of radioiodinated antibody to the basement membrane loops. The background grains in the GBM preparations were also extremely low. To further confirm these observations, combined experiments were performed in which GBMs and isolated glomerular fractions were mixed together and then incubated with the radioiodinated anti-HS-PG antibody. The electron microscopic autoradiograms of Figure 8 A and B reinforced the fact that the anti-HS-PG antibodies do not bind to the glomerular visceral epithelium and that their binding characteristics are exclusively limited to the GBMs. In these micrographs (Fig. 8 A and B), one can readily visualize the binding of the antibody to the GBM, while the adjacent epithelium or its foot processes are devoid of autoradiographic grains.

Discussion

In this investigation, we have provided the data which indicate that the intravenously administered anti-HS-PG antibody binds on either side of the basement membrane initially; with progression of time, it is seen only in the outer layers of the GBM, and, ultimately, the immune deposits are formed in the subepithelial regions where the heterologous antibody remains persistently localized. These data suggest that there *may* be a differential in the clearance of ligands bound in the inner and outer layers of the GBM, with a lag in the latter. Under permissive conditions, that is, availability of antigen:antibody complexation, the subepithelial deposits are formed at the site of trapped ligands.

There are several factors which influence the formation and deposition of immune complex deposits in various regions of

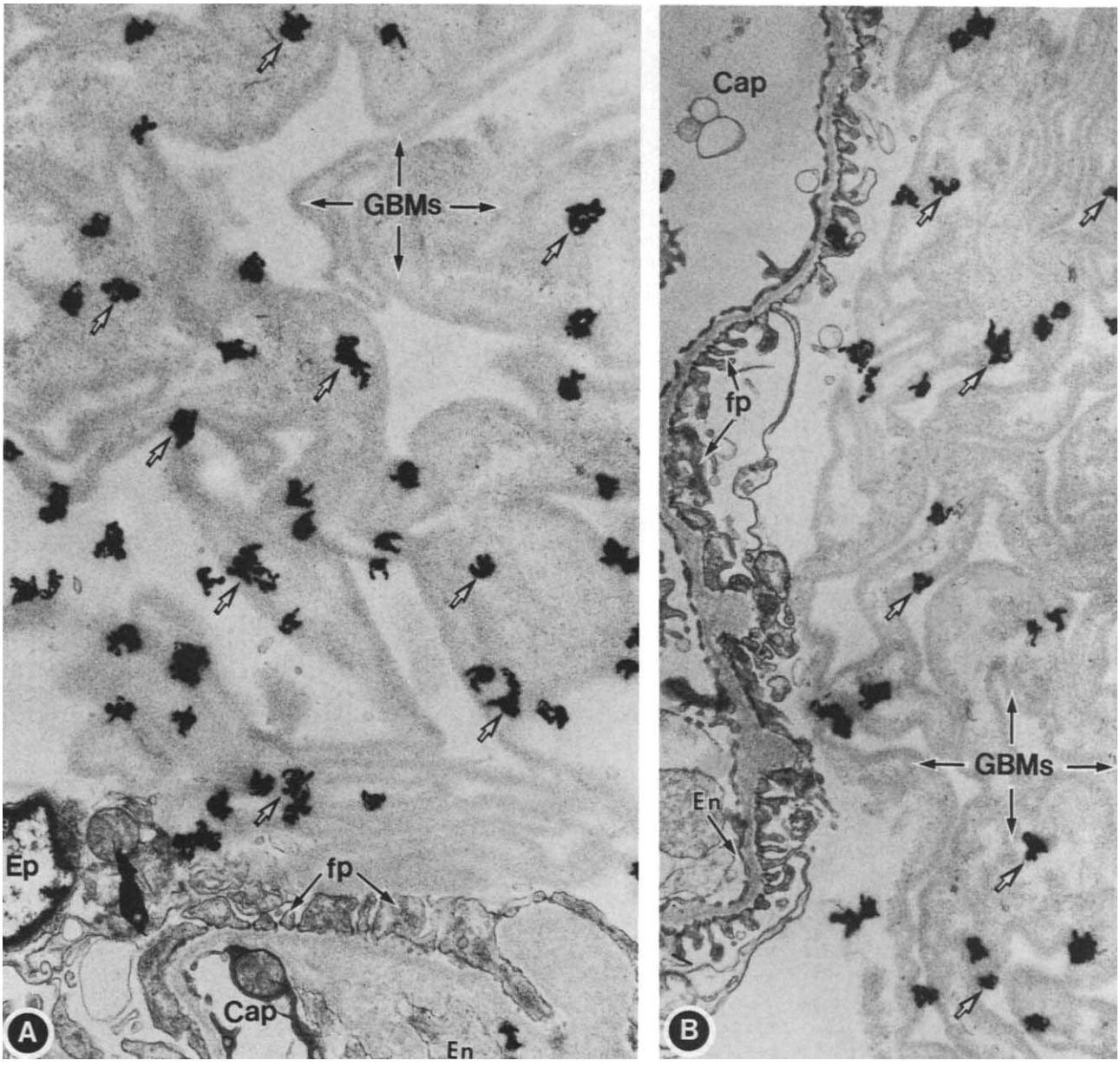

Fig. 8. *Electron microscopic autoradiograms of suspension of isolated glomeruli and GBMs incubated with radioiodinated anti-HS-PG antibody.* Most of the antibody (represented by silver grains and indicated by arrows) is seen bound to the GBM loops. Minimal antibody binding to the epithelium (Ep) and foot processes (fp) is observed. US, urinary space; En, endothelium; Cap, capillary loops. **A**: × 30,000; **B**: × 25,000.

the glomerulus, that is, mesangium, subendothelium, and subepithelium, and these have been the subject of several recent reviews [9, 10, 21–25]. These factors include the size and charge of the antigen, the antibody, and the immune complex; valence of the antigen, the affinity of the antibody, the antigen: antibody ratio, and diffusion coefficient of the complex; and, finally, the intraglomerular hemodynamics, the microanatomy, and the macromolecular composition of the glomerulus. Taking all these factors into consideration, there are two general, fundamental mechanisms which have evolved in the last decade to account for the formation of immune deposits in the glomerular capillary wall. First, the traditional concept indicates that the immune complexes are formed in "circulation" and subsequently are trapped in the basement membrane, forming electron-dense deposits in due course of time [6, 26]. The second, a rather challenging notion with minor variations from model to model, marshals the idea that the antigen:antibody complexes are formed "locally" or *in situ* in the capillary wall [9]. The basic idea for the latter mechanism hinges on the intriguing biochemical/biophysical properties of the glomerular capillary wall, discovered during the middle and late 70's [18, 19, 27–29].

The mechanism of *in situ* immune-complex formation has been investigated mainly in Heymann nephritis, the model of membranous nephropathy in man. In this model, the immune

deposits are seen in the subepithelial region of the glomerulus after two to three weeks of active immunization of the animal with proximal brush border fraction (Fx1A) or its well-characterized purified subfraction, which is gp 330 [30]. Almost similar lesions can be reproduced by infusion or perfusion of antibodies directed against Fx1A or its subfraction gp 330 [31, 32], suggesting that there are certain fixed glomerular antigens with which the corresponding antibodies can interact, and these antigenic sites may very well serve as the nidus for the formation of immune complex deposits ultimately. This concept was strengthened by the experiments carried out with planted non-renal and non-glomerular anionic and cationic antigens [9]. In this regard, the notion of ionic interactions of antigens, antibodies, and immune complexes with the GBM was largely advanced by Gallo et al [22, 33, 34] and followed by Border and his colleagues [24, 35]. In these experiments, the evidence was obtained that the anionic groups of the GBM, enriched with HS-PG, were the pivotal sites for the formation/deposition of immune deposits. Conceivably, in this scenario, the immune complexes are trapped within the GBM by electrostatic interactions or by stereo-specific intercalation between the protein-polysaccharide residues, that is, HS-PG. These stereo-specific and electrostatic interactions are quite captivating; nevertheless, their role in the formation of immune complex deposits remains to be exemplified in a given glomerular disease in man. Also, the role of these exogenous cationic ligands [35–37] in the formation of *in situ* immune-complex is solely dependent upon the electrostatic interactions—the interactions in which other determinants of immune complex formation, that is, van der Waals forces, are excluded. Moreover, the cationic ligands (antigens or antibodies), like other non-immune-complex ligands, may cause structural "damage" to the GBM, thus inducing conformational changes within its macromolecular complex which most likely would lead to spurious localization and formation of *in situ* complexes. Thus, the data available in the literature pertaining to these cationic ligands may be somewhat difficult to interpret and address with respect to the immunobiology in authentic immune-complex nephritis models. On the other hand, the planted antigen-induced glomerular disease, that is, Con A:anti-Con A [38], may have some substantive value in certain immune-complex nephritides in view of the ubiquitous occurrence of these lectins in the environment to which one is constantly exposed. In the present model, the role of ionic interactions is unlikely since the isoelectric point (pI) of the IgG fraction of rabbit anti-HS-PG turned out to be anionic, that is, between 5.5 and 5.8 (unpublished results). In any event, the HS-PG, like certain fractions of Heymann antigen, is an integral constituent of the capillary wall, although residing at a different location, but administration of anti-HS-PG induces identical subepithelial lesions to those seen in Heymann membranous nephropathy. The lesions may be identical; however, the mechanisms in these two models appear to be entirely dissimilar.

The mechanism of selective subepithelial immune-complex deposit formation has been well elucidated by Andres and his colleagues [20, 21]. According to their hypothesis, intravenously administered anti-gp 330 binds to the visceral epithelium, the cell surfaces of which are presumably enriched with gp 330. It is followed by a series of events which includes a firm complexation of antigen:antibody, clustering of the complex, patching, and its shedding into the subepithelial space with the formation of immune deposits ultimately. The key to the whole cascade of events appears to be the homology between gp 330, a subfraction of Fx1A, and the cell surface-associated glycoproteins of the visceral epithelium. In doing so, the interaction of anti-gp 330 and the epithelial membrane of the foot processes apposed to the GBM is conceivable, and the formation of an immune deposit locally is plausible. This hypothesis is quite attractive, but may be exclusive to Heymann nephritis and not applicable to other models of membranous nephropathy. In the anti-HS-PG model, it is unlikely that this sequence of events occurred in view of the data obtained in this investigation. The fact that the binding of anti-HS-PG antibody was exclusive to the GBM, and none with the visceral epithelium, suggests that there may be some other mechanism which may be operational in the pathogenesis of subepithelial deposits in this model. The conceivable sequence of events that one could construct would be as follows: At the outset, the heterologous antibody binds to the lamina rara interna (LRI) and externa (LRE) of the GBM, the regions enriched with HS-PG. The anti-HS-PG antibody is gradually cleared from the LRI by the lateral sweeping plasma water currents causing its dislodgment into the mesangium. The antibody localized in the LRE is not removed readily, which may be due to a decreased shear force in the plasma water currents in this particular region of the GBM. Thus, the antibody in the LRE has a prolonged residence or persistence. The fact that the animal at this given period is in a hyperimmune state, due to presensitization, would lead to a ready production of autologous antibodies. These antibodies would bind to the planted heterologous antibodies, and the formation of electron-dense immune deposits in the subepithelial space ensues. Alternatively, the antigen:antibody complexes (anti-HS-PG:anti-anti-HS-PG) could have formed in circulation, gained access to the LRI, dissociated in the GBM, reaggregated in the LRE, and formed a deposit in the subepithelial space. This latter possibility is unlikely in view of the fact that the heterologous antibody disappears readily from the blood before the animal achieves sufficient titer of the autologous antibody to form immune complexes in circulation. The third possibility that one could conceive would be that the heterologous antibody or immune complexes in LRI were readily removed by the mononuclear phagocytes and the ones in the LRE were inaccessible to them. One may argue against this idea by the fact that PMNs or monocytes were seen in sufficient numbers "in opposition" with the GBM very transiently, while the heterologous antibody was seen in the LRI for a much longer duration. Also during this period, no decrease in the concentration of the antibody in LRE and LRI of isolated glomeruli was observed, which would suggest a less significant role of monocytes or mesangial cells in clearing up the ligands from LRI and an important role of lateral intrinsic fluid currents traversing within the GBM. Thus, the mechanism that one can conceptualize in our model would pertain to the persistent residence of the heterologous antibody in LRE, indirectly related to the differential in the kinetics of plasma currents in various layers of the basement membrane (Fig. 9) which lead to the evolution of subepithelial deposits. Further evidence for the hypothesis of the differential clearance of ligands from various layers of the GBM has also been elucidated in the past by other investigators

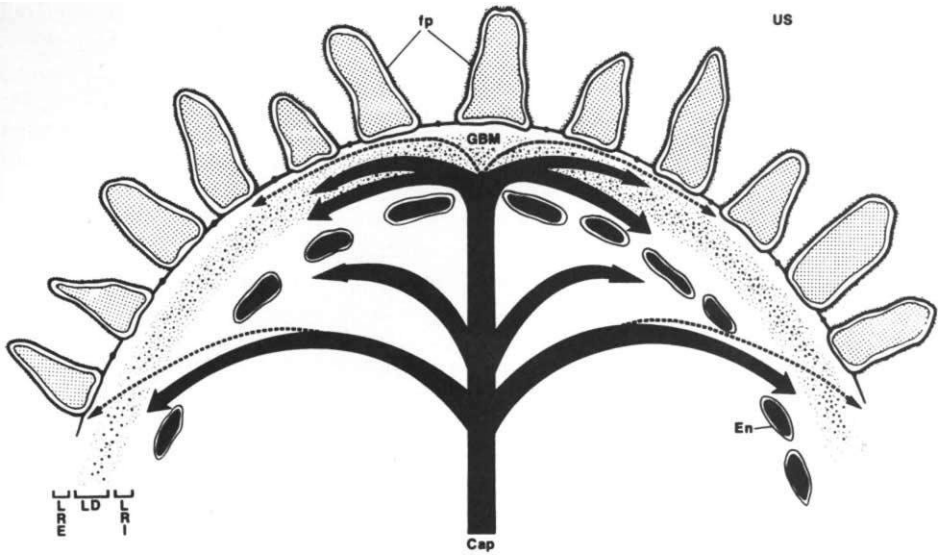

Fig. 9. *Schematic drawing illustrative of kinetics of plasma water currents in various layers of the glomerular basement membrane (GBM).* Normally, the blood/plasma water currents collide vertically with the glomerular capillary wall and are deflected, resulting in the generation of lateral secondary currents. The kinetics of these lateral plasma water currents are strongest at the level of lamina rara interna (LRI, represented by long thick arrows), modest in the lamina densa (LD, represented by medium-sized arrows), and weakest in lamina rara externa (LRE, represented by interrupted thin arrows). The data included in this investigation indicate the possible existence of such currents within the GBM. The currents being strongest in the inner layers, that is, LRI, wash away the immune complexes by virtue of the shear forces and thus the formation of local immune deposits is prevented. While the immune complexes persist in the outer layers, that is, LRE, for a considerable longer period because the shear forces are weak, and this most likely results, under appropriate conditions, in the evolution of subepithelial immune-complex deposits.

[36] with the use of exogenous cationic macromolecules, such as, cationic ferritin.

In conclusion, we have presented another hypothesis which *may* be relevant in the pathogenesis of subepithelial immune deposits in membranous nephropathy.

Acknowledgments

This research was supported by National Institutes of Health Grant AM 28492. Dr. Kanwar is a recipient of Research Career Development Award AM 01018. We are thankful for the secretarial assistance provided by Mary Giles.

Reprint requests to Yashpal S. Kanwar, M.D., Ph.D., Department of Pathology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Illinois 60611, USA.

References

1. Brenner BM, Hostetter TH, Humes HI: Molecular basis of proteinuria of glomerular origin. *N Engl J Med* 298:826–833, 1978
2. Venkatachalam MA, Rennke HG: The structural and molecular basis of glomerular filtration. *Circ Res* 43:337–347, 1978
3. Karnovsky MJ: The ultrastructure of glomerular filtration. *Ann Rev Med* 30:213–224, 1979
4. Farquhar MG, Kanwar YS: Functional organization of the glomerulus, in *Immune Mechanisms in Renal Diseases*, edited by Michael AF, Cumming N, New York, Plenum Publishing, 1982, pp. 1–35
5. Kanwar YS: Biology of disease. Biophysiology of glomerular filtration and proteinuria. *Lab Invest* 51:7–21, 1984
6. Theofilopoulos AN, Dixon FJ: Immune complexes in human diseases. *Am J Pathol* 100:531–591, 1980
7. Spargo BH, Seymour AE, Ordonez MG: In *Renal Biopsy Pathology with Diagnostic and Therapeutic Implications*. New York, John Wiley and Sons, 1980
8. Churg J, Sobin LH: *Renal Disease*. Tokyo, Igaku-Shoin Ltd., 1982
9. Couser WG, Salant DJ: *In situ* immune complex formation and glomerular injury. *Kidney Int* 17:1–13, 1980
10. Heymann W, Hackel DB, Harwood S, Wilson SG, Hunter JP: Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspension. *Proc Soc Exp Biol Med* 100:660–664, 1959
11. Makino H, Lelongt B, Kanwar YS: Nephritogenicity of proteoglycans. II. A model of immune complex nephritis. *Kidney Int* 34:195–208, 1988
12. Unanue ER, Dixon FJ: Experimental glomerulonephritis. VI. The autologous phase of nephrotoxic serum nephritis. *J Exp Med* 121:715–725, 1965
13. Kanwar YS, Jakubowski ML, Rosenzweig LJ: Distribution of sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. *Eur J Cell Biol* 31:290–295, 1983
14. Kanwar YS, Veis A, Kimura JH, Jakubowski ML: Characterization of heparan sulfate-proteoglycan of glomerular basement membranes. *Proc Natl Acad Sci USA* 81:762–766, 1984
15. Roth J, Bendayan M, Carlemalm E, Villinger W, Caravito M: Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J Histochem Cytochem* 29:663–671, 1981
16. Kanwar YS, Rosenzweig LJ, Linker A, Jakubowski ML: Decreased *de novo* synthesis of glomerular proteoglycans in diabetes: Biochemical and autoradiographic evidence. *Proc Natl Acad Sci USA* 80:2272–2275, 1983
17. Krakower CA, Greenspon SA: Localization of the nephrotoxic antigen within the isolated glomerulus. *Arch Pathol* 51:629–639, 1951
18. Kanwar YS, Farquhar MG: Presence of heparan sulfate in the glomerular basement membranes. *Proc Natl Acad Sci USA* 76: 1303–1307, 1979
19. Kanwar YS, Farquhar MG: Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. *Proc Natl Acad Sci USA* 76:4493–4497, 1979

20. CAMUSSI G, BRENTJENS JR, NOBLE B, KERJASCHKI D, MALAVASI F, ROHOLT OA, FARQUHAR MG, ANDRES G: Antibody-induced redistribution of Heymann antigen on the surface of cultured glomerular visceral epithelial cells: Possible role in the pathogenesis of Heymann glomerulonephritis. *J Immunol* 135:2409–2416, 1985
21. ANDRES G, BRENTJENS JR, CALDWELL PRB, CAMUSSI G, MATSUO S: Biology of disease. Formation of immune deposits and disease. *Lab Invest* 55:510–520, 1986
22. GALLO GR, GLASER TC, LAMM ME: Role of electrostatic charge interactions in glomerular deposition of immune complexes. *Pathobiol Annu* 12:203–211, 1982
23. MANNIK M, GAUTHIER VJ: Characteristics of circulating immune-complexes that deposit in renal glomeruli, in *Nephrology, Proceedings of IXth International Congress of Nephrology*, edited by ROBINSON RR, New York, Springer-Verlag Publishing Co., 1985, pp. 527–539
24. BORDER WA: Role of antigen and antibody charge in immune-complex disease, in *Nephrology, Vol. I. Proceedings of IXth International Congress of Nephrology*, edited by ROBINSON RR, New York, Springer-Verlag Publishing Co., 1985, pp. 550–559
25. MICHAEL AF: The glomerular mesangium. *Contr Nephrol* 40:7–16, 1984
26. WILSON CB, DIXON FJ: The renal response to immunological injury, in *The Kidney*, edited by BRENNER BM, RECTOR FC JR, Philadelphia, W.B. Saunders, 1986, pp. 800–889
27. RENNKE HG, COTRAN RS, VENKATACHALAM MA: Role of molecular charge in glomerular permeability. Tracer studies with cationic ferritins. *J Cell Biol* 67:638–646, 1975
28. CAULFIELD JP, FARQUHAR MG: Distribution of anionic sites in the glomerular basement membranes: Their possible role in filtration and attachment. *Proc Natl Acad Sci USA* 73:1646–1650, 1976
29. KEFALIDES NA, ALPER R, CLARK CC: Biochemistry and metabolism of basement membranes. *Int Rev Cytol* 61:167–228, 1979
30. KERJASCHKI D, FARQUHAR MG: The pathogenic antigen of Heymann nephritis is a glycoprotein of renal proximal tubules brush border. *Proc Natl Acad Sci USA* 79:5557–5561, 1982
31. VANDAMME BJC, FLEUREN GJ, BAKKER WW, VERNIER RL, HOEDEMAKKER PH: Experimental glomerulonephritis in rat induced by antibodies directed against tubular antigens. IV. Fixed glomerular antigens in the pathogenesis of heterologous immune-complex nephritis. *Lab Invest* 38:502–510, 1978
32. COUSER WG, STEINMULLER DR, STILMANT MM, SALANT DJ, LOWENSTEIN LM: Experimental glomerulonephritis in the isolated perfused rat kidney. *J Clin Invest* 62:1275–1287, 1978
33. GALLO GR, GLASER TC, LAMM ME: Charge of circulating immune complexes as a factor in glomerular basement membrane localization in mice. *J Clin Invest* 67:1305–1313, 1981
34. CAULIN-GLASER T, GALLO GR, LAMM ME: Nondissociating cationic immune complexes can deposit in glomerular basement membrane. *J Exp Med* 158:1561–1572, 1983
35. ADLER SG, WANG H, WARD HJ, COHEN AH, BORDER WA: Electrical charge. Its role in the pathogenesis and prevention of experimental membranous nephropathy in the rabbit. *J Clin Invest* 71:487–499, 1983
36. VOGT A, ROHRBACH R, SHIMIZU F, TAKAMIYA H, BATSFORD S: Interaction of cationized antigen with rat glomerular basement membrane: *In situ* immune complex formation. *Kidney Int* 22:27–35, 1982
37. GAUTHIER VJ, STRIKER GE, MANNIK M: Glomerular localization of preformed immune complexes prepared with anionic antibodies or with cationic antigens. *Lab Invest* 50:636–644, 1984
38. GOLBUS SM, WILSON CB: Experimental glomerulonephritis induced by *in situ* formation of immune-complexes in glomerular capillary wall. *Kidney Int* 16:148–157, 1979